

Kinetics of phosphorus in *Daphnia* at different food concentrations and carbon : phosphorus ratios

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Abstract

We examined the assimilation efficiency, excretion, and efflux of phosphorus (P) in adults and juveniles of *Daphnia magna* under different food levels (2–40 $\mu\text{g P L}^{-1}$) and dietary carbon : phosphorus (C : P) ratios (90–930 in molar) with *Chlamydomonas reinhardtii* as food. The P assimilation efficiencies calculated by regression analysis were 38–85% and 66–89% for adults and juveniles, respectively, and were constant at food concentrations $>24 \mu\text{g P L}^{-1}$, but increased significantly when the diet shifted from P-sufficiency to P-deficiency. The mass-specific excretion rate of adults and juveniles was 1.1–33.2 $\text{ng P mg dry weight (DW)}^{-1} \text{h}^{-1}$ and 3.0–63.4 $\text{ng P mg DW}^{-1} \text{h}^{-1}$, respectively, and was influenced by the food concentration and decreased with an increase in dietary C : P ratio. The efflux rate constants of the adults and juveniles were 0.182–0.298 d^{-1} and 0.096–0.185 d^{-1} , respectively. Food concentration did not affect the efflux, but an increase in dietary C : P ratio reduced the P efflux, suggesting stoichiometric regulation. Among the different routes involved in P loss from *Daphnia*, molting was the most important, contributing 44–75% of the total loss for the juveniles and adults. The mass specific loss rates were 13–54 $\text{ng P mg}^{-1} \text{h}^{-1}$ and 45–110 $\text{ng P mg}^{-1} \text{h}^{-1}$. The relative and absolute P loss from each compartment (except the dissolved P release in adults) was independent of food concentration. Increasing the dietary C : P ratio decreased the mass-specific release rates by molting, dissolved P release, and reproduction, indicating the animals' endeavor to maintain P stoichiometric homeostasis.

The significance of herbivorous zooplankton in supplying required phosphorus (P) to bacteria and phytoplankton in lake systems has stimulated numerous studies on P releases by different species of zooplankton (Peters and Rigler 1973; Scavia and McFarland 1982; Wen et al. 1994). Very high P excretion by zooplankton has been reported in the literature (e.g., several percent of the total body P content per hour, Lehman, 1980, or as high as 50% of the ingested P, Olsen et al. 1986). Model studies by Peters and Rigler (1973) and Wen et al. (1994) indicated that the P release was generally correlated with the abiotic (e.g., temperature) and allometric parameters. However, direct excretion is not the only pathway in P recycling by zooplankton. Zooplankton can feed on phytoplankton in surface waters and produce sinking fecal materials, which are remineralized by bacteria or contribute to the vertical flux of particulates (Sarnelle 1999). In addition, the P flux associated with living crustaceans may occur by molting, since a considerable percentage of body P is carapace bound (Vrede et al. 1999). Molting of carapace-associated P may represent a substantial drain on the animal's body, e.g., 25 ng of P was lost from moulting in *Daphnia* (Hessen and Rukke 2000). On the other hand, as an intermediate level in the food chain, zooplankton play an important role in the supply of P to higher trophic levels. Thus, P metabolism in herbivorous zooplankton directly affects not

only material transfer to predators but also material input in the microbial loop.

It is well known that *Daphnia* have higher P requirements with higher specific P content than other zooplankton and, thus, suffer more from P limitation in freshwater systems (Hessen 1992; Sterner et al. 1993). In the face of the P-limited but carbon (C)-excessive diets, the animals may improve the P dietary absorption and reduce their C intake or store/dispose the excessive C to maintain a lower body C : P ratio compared with phytoplankton. Regulation of C intake and release has been shown in earlier studies by Demott et al. (1998) and Darchambeau et al. (2003). Demott et al. (1998) found that the P release increased with increasing percentage of P-deficient *Scenedesmus* in the mixture of food, but the P assimilation efficiency did not vary along the dietary C : P gradient. Lehman and Naumoski (1985) also suggested that stoichiometric regulation included the decrease of turnover of structural P under P limitation. However, the exact extent of P limitation is not clear in these studies using a mixture of P-sufficient and P-deficient algae. Besides food quality, food quantity is a common factor affecting P metabolism, but the potential effects have not been considered in these previous studies. Furthermore, since juveniles apparently have higher specific P content and a faster somatic growth rate than do adults (Hessen and Andersen 1991; DeMott 2003), the P assimilation and turnover may be different at different life stages, but again these details also remain less well understood.

Because *Daphnia* are the dominant zooplankton in many freshwater systems, numerous studies have examined their feeding and nutrition, under both laboratory and field conditions, in terms of carbon or energy (Lampert 1975, 1977; Lampert and Bohre 1984; Lynch et al. 1986; Urabe

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Acknowledgments

We thank the two anonymous reviewers for their very constructive comments.

This study was supported by a Competitive Earmarked Research Grant from the Hong Kong Research Grants Council (HKUST6405/05M) to W.-X.W.

Table 1. Food concentration and food quality used in the feeding experiments. Algal species, *C. reinhardtii*; Adu., adult *D. magna*; Juv., juvenile *D. magna*.

Exp.	Treatment	Grazer	Food concentration		C:P ratio in molar
			(mg C L ⁻¹)	(μg P L ⁻¹)	
Food concentration	5 × 10 ³ cells mL ⁻¹	Adu./Juv.	0.07	2	90
	1 × 10 ⁴	Adu./Juv.	0.14	4	90
	2 × 10 ⁴	Juv.	0.28	8	90
	3 × 10 ⁴	Adu./Juv.	0.42	12	90
	6 × 10 ⁴	Adu.	0.84	24	90
	1 × 10 ⁵	Adu./Juv.	1.40	40	90
Food quality	High	Adu./Juv.	0.81	12.0	90
	Medium	Adu./Juv.	0.56	1.8	780
	Low	Adu./Juv.	0.45	2.2	930

and Watanabe 1991). In contrast, there are only sporadic studies on P metabolism (turnover), and the importance of freshwater zooplankton in overall P cycling in freshwater needs further exploration. A detailed budget of P in zooplankton under different ecological conditions is warranted. There is also limited information on the P turnover in *Daphnia* as well as on the dynamic allocation of ingested P into different physiological compartments.

In this study, we employed the radiotracer technique to examine the assimilation efficiency (AE) and turnover rate of phosphorus in *Daphnia magna* at different life stages under different food levels and at different C:P ratios. We used a pure algal culture as food to control the C:P ratio of the cells. We also constructed a detailed integrated budget of phosphorus loss in *D. magna*, including the compartments of excretion of dissolved P, molting, and reproduction, and then assessed the implications of each compartment on the overall phosphorus cycling in freshwater systems. The influences of food concentration, food quality (C:P ratio), and life stage on the budget of P loss were assessed using the mass balance method.

Materials and methods

Algae and zooplankton—The zooplankton *D. magna* was taken from a monoclonal laboratory culture, which was originally obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. In our laboratory, the animals were cultured in glass-fiber (GF/C) filtered pond water at a temperature of 23.5°C with a 14:10 light:dark (LD) cycle for more than 5 years. They were grown on a mixture of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* at saturating food concentration on a daily basis. Because *C. reinhardtii* is the only food species used in all experiments in this study, the animals were fed this single algae for 3 days before the feeding experiments, enabling the animals to acclimate to the food. The algae, *C. reinhardtii* and *S. obliquus*, were separately maintained in an artificial WC medium (Guillard, 1975) for freshwater phytoplankton culture in ultrapure water at 23.5°C, with a 14:10 LD cycle at 100 μmol of photons m⁻² s⁻¹. All experiments were performed at 23°C, and 0.22-μm filtered autoclaved pond water was used to minimize the presence of bacteria in affecting the potential recycling of dissolved phosphorus.

P-limited *C. reinhardtii* were obtained using a chemostat continuous culture, whereas the P-sufficient algae (C:P ratio equal to 90 in molar) were grown in a batch culture with standard WC medium. With a same dilution rate of 0.10 d⁻¹, algae in the continuous culture with different C:P ratios were grown by controlling the P concentrations in the medium. Algal cells with a C:P ratio near 600 and 1,000 were derived from continuous cultures at P concentrations in the medium of 2.5 μmol L⁻¹ and 0.5 μmol L⁻¹, respectively. After at least 5 days of growth, algae were concentrated by centrifugation at 2,000 rpm for 20 min at 15°C. After 7 days, when the algal cells in the batch culture reached the log phase, the cells were concentrated by centrifugation at 3,500 rpm at 15°C for 10 min. All of the algae with three C:P ratios were resuspended in modified WC medium at a P concentration of 1% of the original P concentrations. Afterwards, the carrier-free radioisotope ³³P (as H₃³³PO₄) was spiked at 3.7–5.55 × 10⁶ Bq L⁻¹. After 72 h of radiolabeling, it was assumed that the cells were uniformly labeled with ³³P, with the specific radioactivity in the range of 1.4 × 10⁶ CPM μg⁻¹ P to 3.1 × 10⁶ CPM μg⁻¹ P. The radiolabeled algae were collected by centrifugation again and added to GF/C filtered pond water at different concentrations before being fed to the *Daphnia*. At the same time, the C and P contents were measured, and the final molar C:P ratios of the algae were measured.

Assimilation, excretion, and ingestion rate—Two experiments were performed to test the influences of, respectively, food concentration and C:P ratio on the dietary P AE in juvenile and adult daphnids (Table 1). In the food concentration experiment, P-sufficient algal cells were used as food (C:P = 90 in molar). Five different treatments were included (5 × 10³, 1 × 10⁴, 2–3 × 10⁴, 3–6 × 10⁴, and 1 × 10⁵ cells mL⁻¹, corresponding to 2, 4, 8–12, 12–24, and 40 μg P L⁻¹ and 0.07, 0.14, 0.28–0.42, 0.42–0.84, and 1.4 mg C L⁻¹) for both juveniles (4–5 days old) and adults (11–13 days old). In the C:P ratio experiment, three different treatments were included (C:P ratios of 90, 780, and 930, as high, medium, and low P treatments, respectively). The food concentration used in this experiment was 3 × 10⁴ cells mL⁻¹ for both juveniles (4–5 days old) and adults (13–14 days old). There were three

replicated bottles for each food condition treatment. Before the pulse-chase feeding, adults were fed *C. reinhardtii* for 3 days under corresponding food conditions. The animals with similar body sizes were then starved for 3 h without food to evacuate the materials in the guts. During the pulse-feeding, 32 adults or 35 juveniles were fed radiolabeled algae in 160–175-mL pond water at different food conditions for 7 min, which was shorter than the minimum gut passage time, to avoid the defecation of radioactive feces during the feeding period. After the pulse feeding, the animals were removed with a mesh, and rinsed with 0.22- μm filtered pond water. Four adults or five juveniles were immediately removed from each replicate bottle and transferred into vials containing 0.5 mL of 1 mol L⁻¹ NaOH (used to solubilize the animals) for later radioactivity counting, which was regarded as the total amount of radioactivity ingested by *Daphnia* during the pulse feeding period. For each bottle, the remaining 28 adults and 30 juveniles were transferred into 150 mL and 100 mL of new pond water, respectively, to depurate their ingested radiolabeled materials in the presence of nonradioactive food under the same conditions. The water was changed after 1, 2, 5, 9, 12, and 24 h to minimize the occurrence of ³³P-recycling. Each time the water was changed, 5 mL of water aliquot was pipetted into the plastic vials for later measurements of total phosphorus (as T³³P), and another 5 mL was filtrated through 0.22 μm Millipore membrane to remove the particulate materials before counting the dissolved phosphorus (as D³³P). Meanwhile, four adults or five juveniles from each replicated bottle were transferred to the vials for later radioactivity counting.

During the experiments, the animals were handled gently to minimize any damage to the animals and loss of ³³P. All procedures were conducted immediately to avoid the utilization of ³³P by the algae. During the depuration period, the animals that shed molts were discarded.

To determine the gut passage time (GPT) of the food particles, the animals were first evacuated of gut contents for 2 h in the absence of food particles, and each individual was then placed in a well dish containing 10 mL of filtered pond water with different food concentrations. Any producing feces was observed continuously under the microscope. The GPT is the time required for the first appearance of feces.

Efflux and loss budget—Two sets of experiments were also performed to test the influences of food quantity and C:P ratio on the P efflux of the animals. In the food quantity experiments, 50 adults (10–11 days old) or 60 neonates (younger than 24 hours) in each bottle (240–350 mL pond water) were fed the ³³P-radiolabeled P-sufficient *C. reinhardtii* (C:P = 90 in molar) at a cell density of 1–4 $\times 10^4$ cells mL⁻¹ (0.27–1.1 mg C L⁻¹) for a total of 3 days. Every 12 h, the animals were fed radioactive cells for 2 h. After being rinsed with 0.22- μm filtered pond water, they were transferred into new pond water and fed nonradioactive food for the remaining 10 h. This radiolabeling process lasted for 3 days to ensure that the structural pool was radiolabeled with ³³P. Adults or juveniles were then collected and, after being rinsed with

filtered pond water, several individuals were removed for measurement of the initial radioactivity retained in the animals (before the depuration). The remaining 44 adults and 55 juveniles were subsequently depurated in 220–310 mL of filtered pond water, with the addition of nonradioactive food (*C. reinhardtii*) at three different concentrations (5 $\times 10^3$, 3 $\times 10^4$, and 10⁵ cells mL⁻¹ or 0.07, 0.42, and 1.4 mg C L⁻¹) for 4 days. There were three replicates for each treatment. During the 4 days of depuration, to minimize the potential recycling of ³³P by the daphnids (because of rapid uptake of regenerated ³³P by the algae), the animals were fed food at corresponding concentrations for 3 h and were transferred to new filtered pond water without food for 9 h before the water renewal (i.e., the water was changed every 12 h, and the daphnids were fed two times each day).

In the C:P ratio experiments, radiolabeling procedures of the adults and juveniles were almost the same as the food quantity experiments, except that animals were pre-fed ³³P-radiolabeled *C. reinhardtii* at different C:P ratios, 90, 780, and 930, at food levels of 1–4 $\times 10^4$ cells mL⁻¹. Similar to the food quantity experiments, the initial radioactivity of the animals was measured at the end of labeling, and the remaining 44 adults and 55 juveniles were subsequently depurated in 220–310 mL filtered pond water. During the 3 days of depuration, *Daphnia* in these three treatments were fed nonradioactive food with corresponding C:P ratios. In this experiment, the animals were fed food at 2 $\times 10^5$ cells mL⁻¹ (with the corresponding C:P ratios) for 2 h and were transferred to new filtered pond water without food for 6 h before the water renewal (i.e., the water was changed every 8 h, and the daphnids were fed three times each day). It was assumed that re-uptake of excreted dissolved phosphorus (DP) by the P-limited algae occurred quicker than by the P-sufficient algae.

Before the water renewal in both groups of experiments, the daphnids (two adults or three juveniles) were removed for the measurement of radioactivity as described above for the short-term experiments. Molts and neonates produced by the adults were carefully collected with a wide mouth pipette. The short interval of measurement (every 12 h or 8 h) ensured that any molts were newly originated from the adults within the 12 h or 8 h. A 5-mL subsample was also removed each time the medium was changed for measurements of dissolved phosphorus (D³³P).

To calculate the specific radioactivity of the animals, both the efflux rate of ³³P and the growth rates of animals were required. Another set of experiments was therefore performed to measure the somatic growth rates of adults and neonates for all of the treatments. About 100 adults or 150 juveniles in each treatment were grown with food under the same conditions as those in the efflux experiments, except that the nonradioactive food particles were used instead. Ten adults or 15 juveniles were collected at 0, 24, 48, and 72 h to measure the P contents in the animals. The somatic growth rates were calculated as the slope of regression between the natural log of P against the time of growth.

Chemical and data analysis—Carbon contents of algae and *Daphnia* were measured with a CNH analyzer (Series II

CHNS/O Analyzer 2400, PerkinElmer Instruments), and P contents were measured by molybdate blue reduction (Murphy and Riley 1962) after hot acidic oxidative hydrolysis with 5% $K_2S_2O_8$ after the tissues were dissolved with hydrogen peroxide at 130°C. Algal cells were collected by filtration onto precombusted (500°C, 4 h) GF filters and were dried at 80°C for 2 days. *Daphnia* for both elemental and dry-weight measurements were dried at 80°C for 2 days, and the dry weights were determined.

The animals were solubilized in 0.5 mL 1 mol L⁻¹ NaOH at 60°C overnight. Subsequently, a 6-mL cocktail (Perkin Elmer) was added, and the radioactivity was counted. ³³P was measured by a Wallac 1414 liquid scintillation counter using the external standard ratio method. Counting times were adjusted to yield a propagated counting error of <5% (typically 2–3%).

The ingestion rate was calculated using the specific radioactivity of food particles and the radioactivity of *Daphnia* at the end of pulse-feeding. Following Penry (1998), assimilation efficiency in this present study was defined as the fraction of digestive products that is taken up across the cell membranes of the gut wall. Our results showed that ³³P lost by feces egestion accounted for 85–100% of total body ³³P loss within the first 2 h of depuration. Afterward, P excretion dominated the overall P loss from the animals. AE was therefore calculated as the y-intercept of the linear regression between the natural log of percent of P retained in the animals and the time of depuration (2–24 h) (Wang and Fisher 1999). The relative excretion was calculated as DP/ingestion using the sum of D³³P during 2–24 h in the short-time incubation. The excretion rate was calculated by multiplying the relative excretion rate and the ingestion rate.

The percentage of P released into each compartment was calculated as the radioactivity detected in each compartment divided by that in the total phosphorus (T³³P). In the short-term experiments, T³³P was the sum of radioactivity in DP and feces, whereas it was the sum of ³³P in all compartments (DP, molts, and neonates) in the long-term experiments. Generally, the fraction of D³³P absorbed onto the container walls accounted for <1% of D³³P produced during the experiment. The average specific radioactivity of animals during a certain interval (12 h or 8 h) in the efflux experiments was calculated by the following equation:

$$\bar{S} = \left(\int_{t_1}^{t_2} S_0 \cdot e^{-(k+g)\Delta t} \right) / \Delta t \quad (1)$$

Where, t_x is the time point of sampling, S_0 is the initial specific activity, k is the efflux constant rate, and g is the specific growth rate of the animals.

Statistical analysis—One-way analysis of variance (ANOVA) was performed to compare AE, efflux constant rate, relative distribution of P loss, and mass-specific loss rate at different food concentrations and dietary C:P ratios for a certain life stage, followed by Tukey post-hoc test. To meet the assumption of normality for an ANOVA, the percentage data were arcsin transformed, and natural log

transformations were applied to other data where appropriate, before the analysis. The level of significance for all tests was $\alpha = 0.05$. Contribution of life stage and interaction between food concentration or dietary C:P ratio and life stage to AE, efflux constant rate, relative distribution of P loss, and mass-specific loss rate were analyzed by analysis of covariance (ANCOVA).

Results

Assimilation and ingestion—In both short- and long-term experiments, the sum of radioactivity in each compartment and P loss from the animals was compared. The mass balance of the lost P was generally in the range of 95–115%.

After 7 min of radioactive pulse-feeding, the phosphorus was lost from *Daphnia* rapidly within 1–2 h. Afterward, the lost become gradual (Fig. 1). Both adults and juveniles had higher P AEs at lower food concentrations (Table 2). Statistical analysis showed that the food concentration significantly affected the AEs, especially for the adults (juveniles, $F_{4,10} = 62.5$, $p < 0.01$; adults, $F_{4,10} = 244.0$, $p < 0.01$). The relationship between P AE and food concentration is best described by an exponential equation (Fig. 2). The juveniles had P AE higher than the adults at the same food concentrations, and ANCOVA analysis verified the significant effect of life stage on AE ($F_{1,19} = 37.1$, $p < 0.05$). In experiments with the C:P ratio, the pattern for P loss from *Daphnia* was similar to that in the food concentration experiments (Fig. 1). Both adults and juveniles fed low-quality food (C:P = 930 and 780) had 20% higher phosphorus AE than had those fed high-quality food (One-way ANOVA, adults, $F_{2,6} = 117.8$, $p < 0.01$; juveniles, $F_{2,6} = 277.1$, $p < 0.01$). The AE did not differ between the two low-quality treatments (Tukey post-hoc test). At the same C:P ratios, the AE was also significantly higher in the juveniles than in the adults by 70% (Table 2) (ANCOVA, $F_{1,14} = 514.4$, $p < 0.01$).

The ingestion rate (IR) increased with an initial increase in food concentration and then leveled off (Table 2). The incipient limiting food concentration was around 12 $\mu\text{g P l}^{-1}$ or 0.42 mg C L⁻¹ (3×10^4 cells mL⁻¹). *Daphnids* fed P-deficient algae had significantly lower IRs in terms of P content than had those fed P-sufficient food (Table 2) (One-way ANOVA, adults, $F_{2,6} = 180.8$, $p < 0.001$; juveniles, $F_{2,6} = 277.6$, $p < 0.001$). The difference in the amount of food particles ingested among the treatments was less obvious (but still statistically significant) than the P IR. The difference in P IR was thus primarily caused by the different P contents of the food particles.

The percentages of P released as dissolved phosphorus during the 24-h (0–24 h) depuration period are shown in Fig. 3. For adults, dissolved excretion accounted for 35% of the total phosphorus loss at the three low food concentrations. At the two high food concentrations, the relative contribution of dissolved excretion decreased to 15–20%. There was a significant log negative correlation between the percentage of DP released into the dissolved phase and the food concentration ($r^2 = 0.95$, $p < 0.01$ for adults; $r^2 = 0.80$, $p < 0.05$ for juveniles). For both juveniles and adults fed high-quality food, dissolved phosphorus

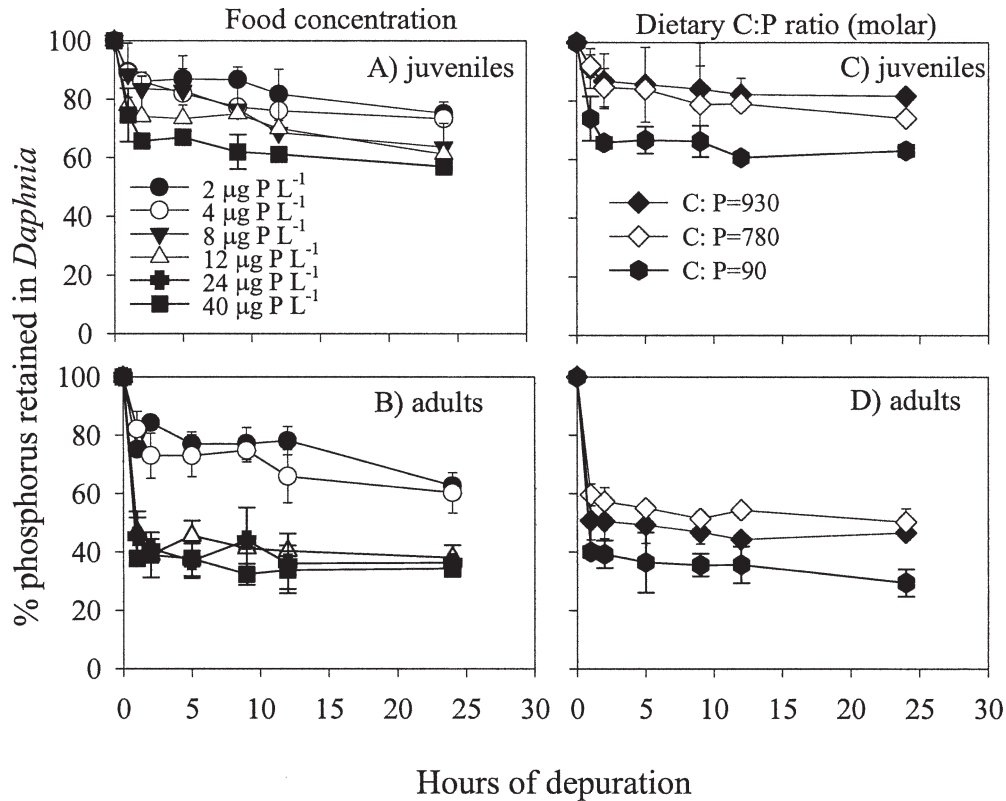


Fig. 1. Retention of ingested P in adults and juveniles of *D. magna* after a pulse ingestion of radiolabeled *C. reinhardtii* at different food concentrations (A, B) and different dietary C:P ratios (C, D). Data are means \pm SD ($n = 3$).

contributed more to the total P loss than for those fed low-quality food (One-way ANOVA, adults, $F_{2,6} = 108.1$, $p < 0.05$; juveniles, $F_{2,6} = 181.3$, $p < 0.01$).

During the 24-h deputation period, the fraction of ingested P released by direct excretion was typically small ($< 11\%$, Table 2) based on the accumulation of dissolved labeled P from 2–24 h. Because the P lost by molting was excluded during the short-term deputation, the mass-specific P excretion from the freshly incorporated tissues was also calculated based on the dry weights of the animals (0.11–0.19 mg and 0.22–0.28 mg for the 5–6-day-old juveniles and 12–14-day-old adults, respectively, He and Wang, unpubl. data) (Table 2). Mass-specific P excretion rates differed among different food concentration treatments (adults, $F_{4,10} = 15.4$, $p < 0.01$; juveniles, $F_{4,10} = 1331.7$, $p < 0.01$). Interestingly, the highest excretion rate for both adults and juveniles was found at the medium food concentration ($2\text{--}3 \times 10^4$ cells mL^{-1} , close to the incipient food concentration). When the food shifted from P-sufficient to P-deficient, the average excretion rate decreased by 6–19-fold (adults, $F_{2,6} = 164.8$, $p < 0.01$; juveniles, $F_{2,6} = 17658.2$, $p < 0.01$). At the same dietary C:P ratio (for 90 and 780), the juveniles had a significantly higher excretion rate than did the adults (Table 2).

Efflux and P budget—Phosphorus was lost from *Daphnia* at a comparable rate throughout the 4-day deputation period at different food concentrations (Fig. 4). At the end

of deputation, 46–54% and 36–42% of P remained in the juveniles and adults, respectively. The relationship between phosphorus retained in *Daphnia* and deputation time was well fitted by a one-compartment model ($p < 0.01$). Thus, the efflux rate constants of P were calculated from the linear regression between the natural log of P retained in the animals and the time of deputation (one day onward, Table 3). The efflux rates were comparable at different food concentrations for each life stage (adults, $F_{2,6} = 1.0$, $p = 0.407$; juveniles, $F_{2,6} = 2.6$, $p = 0.156$), and juveniles had lower efflux rates than adults, although the difference was not statistically significant (ANCOVA, $F_{1,14} = 2.2$, $p = 0.161$).

In the food quality experiments, the loss of body phosphorus from *Daphnia* was also gradual during the 3-day deputation period. After 72 h, 57–68% and 46–52% of phosphorus remained in the juveniles and adults, respectively. Similarly, the efflux rate constants were calculated by regression analysis (Table 3). With a shift in diet from P-deficient (C:P ratio > 300) to P-sufficient (C:P ratio < 300), there was a significant increase of the efflux rate constant for both adults and juveniles (One-way ANOVA, adults, $F_{2,6} = 7.2$, $p = 0.025$; juveniles, $F_{2,6} = 4.4$, $p = 0.067$), and the efflux rates in juveniles were significantly lower than those in adults (ANCOVA, $F_{1,14} = 33.3$, $p < 0.01$).

The averaged contribution of each loss route (molting, release of DP, and neonate production) to the overall P loss

Table 2. Assimilation efficiency (AE), gut passage time (GPT), fraction of ingested P directly excreted (as DP/ingested P, %), ingestion rate, and mass-specific excretion rate of phosphorus in the adults and juveniles of *Daphnia* feeding at different food conditions. Mean \pm SD ($n = 6$ for GPT and $n = 3$ for other parameters).

Treatments	AE (%)		GPT (min)		DP/ingested P (%)		Ingestion rate ($\times 10^3$ cells ind $^{-1}$ min $^{-1}$)		Excretion rate (ng P mg $^{-1}$ h $^{-1}$)	
	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles
Food concentration ($\mu\text{g P L}^{-1}$)										
2	85.4 \pm 2.7	89.2 \pm 1.6	27.0 \pm 6.0	35.0 \pm 7.0	3.51 \pm 1.14	5.92 \pm 1.38	2.47 \pm 0.47	0.86 \pm 0.28	0.99 \pm 0.19	0.35 \pm 0.11
4	76.4 \pm 2.6	84.8 \pm 1.9	16.0 \pm 2.0	15.7 \pm 1.2	5.23 \pm 1.08	6.06 \pm 1.64	3.68 \pm 0.94	1.90 \pm 0.22	1.43 \pm 0.38	0.76 \pm 0.09
8	nd	86.1 \pm 2.6	nd	10.0 \pm 5.2	nd	10.77 \pm 1.85	nd	2.66 \pm 0.36	nd	1.06 \pm 0.14
12	42.6 \pm 2.3	77.3 \pm 2.0	6.0 \pm 1.0	8.7 \pm 4.0	4.26 \pm 0.36	5.22 \pm 1.36	8.84 \pm 0.43	4.35 \pm 0.40	3.54 \pm 0.17	1.74 \pm 0.16
24	40.9 \pm 2.8	nd	7.0 \pm 2.0	nd	2.49 \pm 0.14	nd	10.48 \pm 2.11	nd	4.19 \pm 0.84	nd
40	37.5 \pm 2.0	67.4 \pm 1.2	5.0 \pm 0.3	7.0 \pm 2.0	1.90 \pm 0.74	2.00 \pm 0.25	11.02 \pm 3.75	5.16 \pm 1.31	4.41 \pm 1.5	2.06 \pm 0.52
C:P ratio										
930	49.4 \pm 1.7	86.5 \pm 0.8	nd	nd	3.80 \pm 1.26	4.88 \pm 2.93	2.28 \pm 0.46	0.52 \pm 0.25	0.14 \pm 0.10	0.03 \pm 0.02
780	56.7 \pm 1.4	85.3 \pm 1.1	nd	nd	1.60 \pm 0.64	4.97 \pm 1.35	5.67 \pm 1.62	1.86 \pm 0.23	0.42 \pm 0.06	0.14 \pm 0.02
90	39.8 \pm 0.8	66.3 \pm 1.8	nd	nd	2.70 \pm 0.46	5.91 \pm 0.58	7.12 \pm 0.79	4.04 \pm 0.38	3.06 \pm 0.34	1.74 \pm 0.17

nd, not determined.

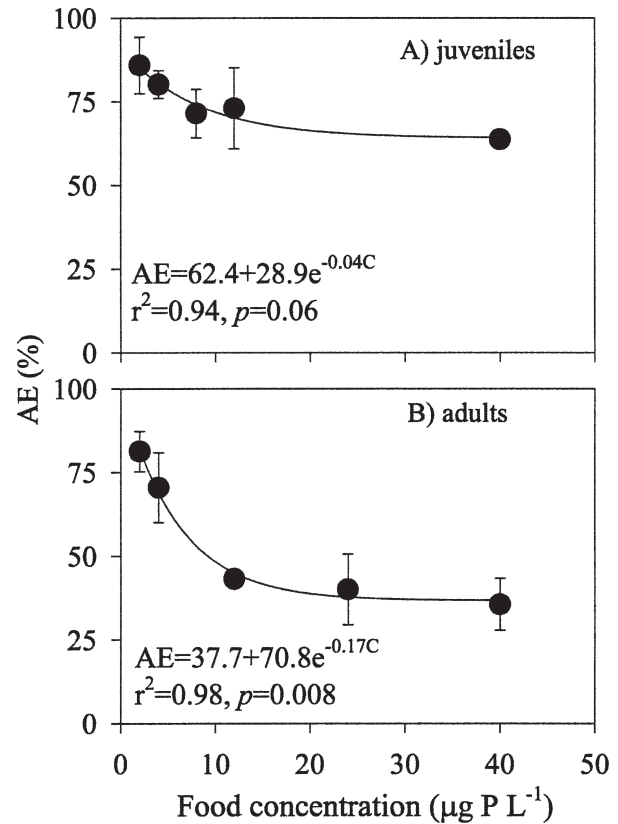


Fig. 2. The relationship between the P AE and the food concentration in (A) juveniles and (B) adults of *D. magna*. Data are means \pm SD ($n = 3$).

from *Daphnia* was calculated by integrating the entire depuration period under different food levels and qualities (Fig. 5). Absolute loss from *Daphnia* was calculated by dividing the radioactivity of each compartment at each time point with the corresponding specific radioactivity of the animals calculated by Eq. 1, and the mass-specific loss rate was calculated by dividing the absolute loss with the average dry weight and depuration time (Fig. 6). Molting and DP release accounted in juveniles for 66–75% and 25–34% of the total P loss during the entire depuration period, respectively, at different food concentrations. The calculated specific loss rate was 46–54 ng mg DW $^{-1}$ h $^{-1}$ and 19–26 ng mg DW $^{-1}$ h $^{-1}$, respectively. In adults, molting represented the major route for the P drain from the daphnids' bodies, followed by neonate production and release of dissolved phosphorus. During the entire depuration period, each adult, on average, allocated 1.72–1.92 $\mu\text{g P}$, 0.98–1.47 $\mu\text{g P}$, and 0.52–1.16 $\mu\text{g P}$ into molting, neonate production, and DP release, with a corresponding specific loss rate of 88–98 ng mg DW $^{-1}$ h $^{-1}$, 50–75 ng mg DW $^{-1}$ h $^{-1}$, and 27–59 ng mg DW $^{-1}$ h $^{-1}$, respectively. There was no significant difference in the relative importance of different P loss routes between juveniles and adults. Food concentration did not cause a difference in the relative contribution of each of these compartments, except that increasing the food concentration resulted in an increase in the relative DP contribution in the adults

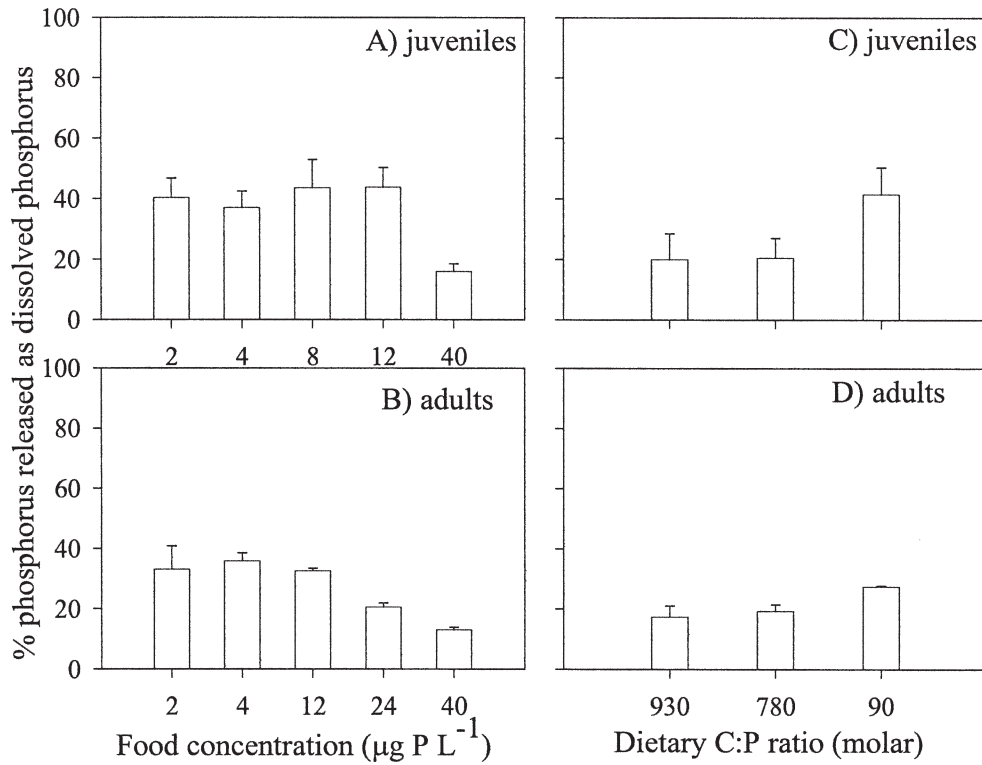


Fig. 3. Relative contributions of dissolved P release from adults and juveniles of *D. magna* fed *C. reinhardtii* during the 24-h depuration period at different food concentrations (A, B) and dietary C:P ratios (C, D). Data are means \pm SD ($n = 3$).

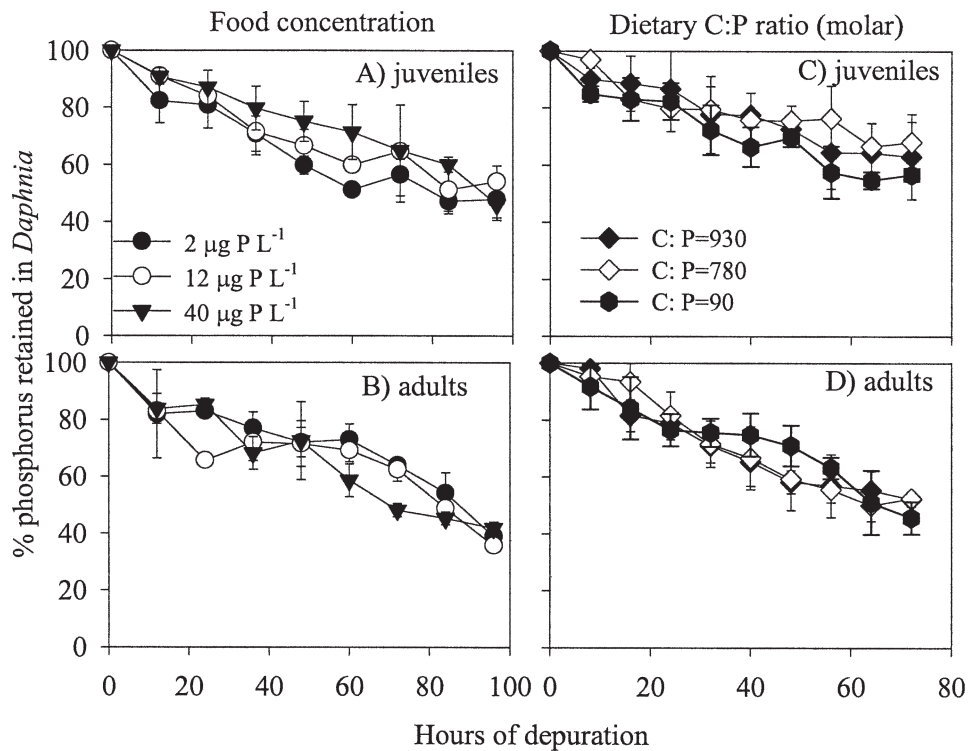


Fig. 4. Retention of P in the radiolabeled juveniles and adults of *D. magna* at different food conditions (A, B) and dietary C:P ratios (C, D) fed *C. reinhardtii* during the 3–4 days of depuration. Data are means \pm SD ($n = 3$).

Table 3. Efflux rate constant (k) of phosphorus in *D. magna* fed *C. reinhardtii* at different food conditions. Mean \pm SD ($n = 3$).

Treatments	k (d^{-1})	
	Adults	Juveniles
Food concentration ($\mu\text{g P L}^{-1}$)		
2	0.216 \pm 0.050	0.156 \pm 0.038
12	0.228 \pm 0.060	0.120 \pm 0.031
40	0.228 \pm 0.029	0.185 \pm 0.036
C:P ratio		
930	0.182 \pm 0.024	0.125 \pm 0.024
780	0.221 \pm 0.031	0.096 \pm 0.026
90	0.298 \pm 0.053	0.173 \pm 0.043

(Table 4). The mass-specific loss rate of DP excretion increased with increasing food level, whereas the difference in loss rate by neonate release occurred only between the lowest and the highest food level (Table 4, Tukey post-hoc test).

In the C:P ratio experiment, molts and DP, respectively, accounted for 50–63% and 37–50% of total P loss in juveniles and 44–54% and 15–33% of the total loss in adults (Fig. 5). Neonates accounted for 14–35% of total loss. Different food qualities did not result in different allocations of P loss (%) in juveniles. However, in the adults, more P was lost through DP release in the low quality treatments (C:P = 780 and 930), whereas with increasing C:P ratio, less P was released by reproduction (Table 4).

Obviously, there was a shift of importance between DP and reproduction in adults when the dietary C:P increased from below to above the threshold (C:P ratio = 300 was generally considered as the threshold from food saturation to P-limitation). Specifically, juveniles lost P at rates of 13–46 $\text{ng mg}^{-1} \text{h}^{-1}$ and 11–21 $\text{ng mg}^{-1} \text{h}^{-1}$ through molting and DP release, respectively. The specific loss rates for the adults were 45–110 $\text{ng mg DW}^{-1} \text{h}^{-1}$, 15–77 $\text{ng mg DW}^{-1} \text{h}^{-1}$, and 27–33 $\text{ng mg DW}^{-1} \text{h}^{-1}$ in molts, neonates, and DP, respectively (Fig. 6). Feeding with different P-content food caused a difference in mass-specific loss rates through all of the compartments (Table 4). The two low-quality treatments (C:P ratios of 930 and 780) had rough similarity in their loss rates, whereas the P loss through molts and reproduction increased significantly at a C:P ratio of 90. Furthermore, the P loss rates were higher in the adults than the neonates (ANCOVA, moult, $F_{14,1} = 48.7$, $p < 0.001$; DP, $F_{14,1} = 102.4$, $p < 0.001$).

Discussion

Assimilation efficiency and excretion—The P AEs were 38–85% and 66–89%, respectively, for adults and juveniles, within the food levels examined in this study. Since the influences of food concentration and quality on phosphorus AE have not been commonly studied, very little information can be obtained from the literature for comparisons with our values. Peters and Rigler (1973) quantified the flux of ^{32}P in *Daphnia rosea* feeding on the yeast *Rhodotorula*. They used the equation assimilation =

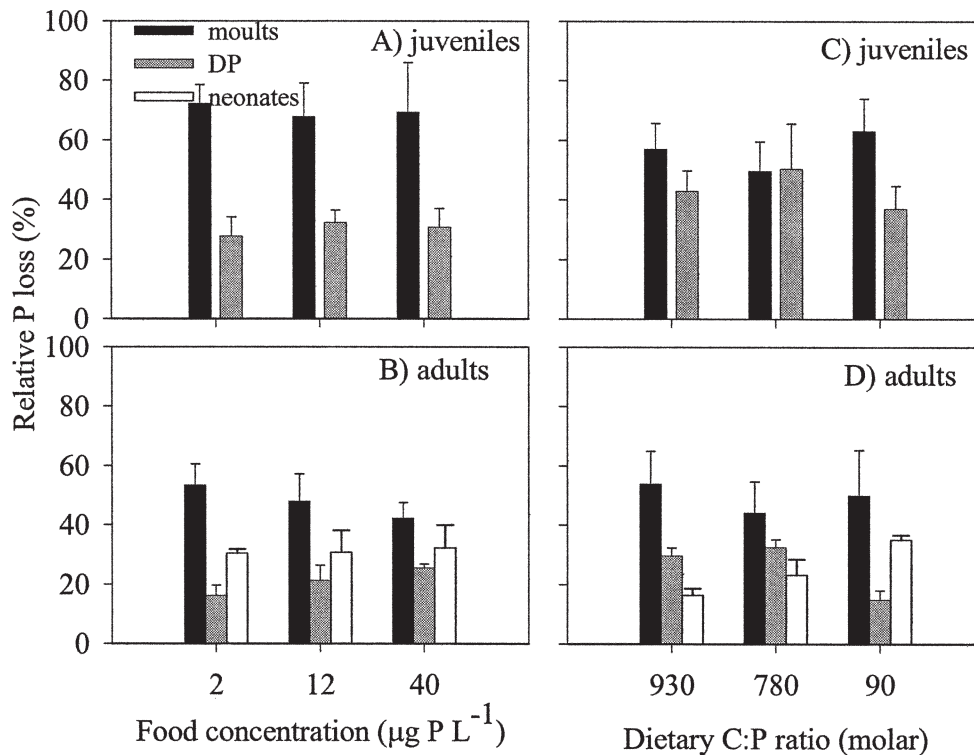


Fig. 5. The relative contribution of different routes of P loss from the adults and juveniles of *D. magna* during the 3–4 days of depuration fed *C. reinhardtii* at different food concentrations (A, B) and at different dietary C:P ratios (C, D). Data are means \pm SD ($n = 3$).

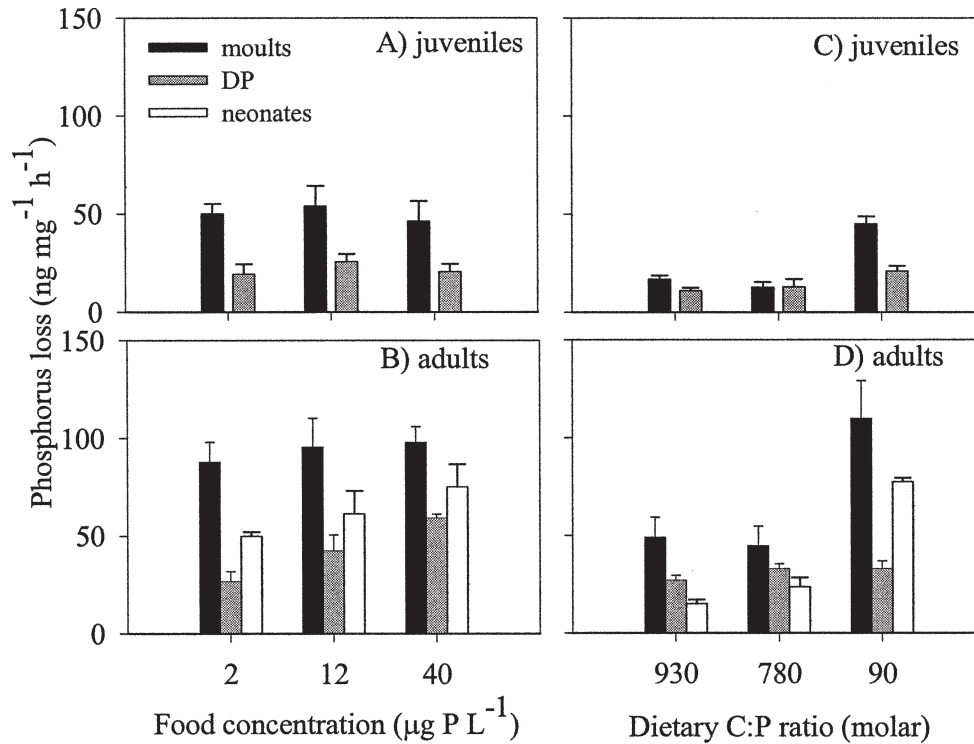


Fig. 6. The mass-specific loss rate by different routes from the adults and juveniles of *D. magna* during the 3–4 days of depuration fed *C. reinhardtii* at different food concentrations (A, B) and dietary C:P ratios (C, D). Data are means \pm SD ($n = 3$).

growth + reproduction + excretion to obtain an average AE of 54%, but with a rather large variability. Hessen and Andersen (1990) stated that *Daphnia* retained nearly 82% of ingested P from bacteria in a labeled seston. Wen et al. (1994) modeled the phosphorus fluxes in six species of limnetic cladocerans and found a P AE generally above 60%. These literature values were somewhat comparable with our measurements, but it should be emphasized that different approaches were used in different studies in quantifying or calculating the P AEs.

Relationships between the food concentration and the ingestion rate were similar to those typically found in the literature (Lampert 1987). In our study, the incipient

limiting level (ILL) was about $24 \mu\text{g P L}^{-1}$ (equivalent to 0.8 mg C L^{-1}), which was at the high end generally found among different *Daphnia* species (i.e., Geller 1975; Kersting and Van der Leeuw 1976; Porter et al. 1982). In both juveniles and adults, an increase in food concentration was accompanied by a decrease in P AE, and at food concentrations $>24 \mu\text{g P L}^{-1}$, the AE become constant, suggesting the occurrence of typical superfluous feeding. Additional evidence of superfluous feeding was found in the negative correlation between the GPT (5–27 min for the adults and 7–35 min for the juveniles) and the food concentration in *Daphnia* (Table 2). The somewhat higher ILL may be related to the 3 h of starvation before the

Table 4. Summary table of the one-way ANOVA applied to the relative distribution of P loss and specific loss rate through different pathways during the efflux period in adult and juvenile *D. magna*.

Experiment	Life-stage	Compartments	df	Relative distribution of P loss		Specific loss rate	
				$F_{2,6}$	p	$F_{2,6}$	p
Food concentration	Adults	Moults	2	1.5	0.292	0.7	0.55
		DP	2	6.3	0.033	24.6	0.001
		Neonate	2	0.1	0.946	5.1	0.05
	Juveniles	Moults	2	1.2	0.356	0.6	0.60
		DP	2	2.7	0.146	1.8	0.24
Food C:P ratio	Adults	Moults	2	0.5	0.630	21.3	0.002
		DP	2	37.1	<0.001	4.4	0.066
		Neonate	2	27.4	0.001	346.6	<0.001
	Juveniles	Moults	2	1.4	0.320	26.0	0.001
		DP	2	1.5	0.306	7.2	0.026

feeding experiment. Earlier studies showed that after starvation, the feeding rate did not exhibit a typical feeding curve but continued to increase with food level above the usual ILL (McMahon and Rigler 1965; Geller 1975).

In addition to food quantity, food quality is another potential factor influencing the efficiency of P utilization. Generally, low food quality (high C:P ratio) caused an increase in AE in our study. On the contrary, Demott et al. (1998) found a decline in the P AE along the gradient of P deficiency using a mixture of P+ and P- *Scenedesmus* as food. In their study, assimilation efficiency (net incorporation + excretion) was calculated as the fraction of P retained in *Daphnia* after 40 min of gut clearance after 10 min of feeding. In our study, the ingested food took 1–2 h before complete egestion, thus the AE may be overestimated if calculated with a shorter period of clearance. Furthermore, it was noticeable that with the gradient of P deficiency, the rate by which P was released through defecation decreased, which may explain the difference between Demott et al. (1998) and our present study. We also found that there was a negative relationship between ingestion rate and dietary C:P ratio. Earlier studies also reported a decrease in absolute ingestion or clearance rate with a rise in the food C:P ratio (Sterner et al. 1993, Van Donk and Hessen 1993, DeMott et al. 1998), but other studies found a constant clearance rate (e.g., Van Donk et al. 1997; Hessen et al. 2002; Darchambeau et al. 2003). Plath and Maarten (2001) directly measured the appendage beating rate and found an increasing feeding activity with an increase in the C:P ratio of the algae. They suggested that the feeding behavior in *Daphnia* was sensitive to food quality and the feeding activity may be altered to maintain the homeostasis of the animals. An increasing filtration rate when the animals encountered low-quality food in situ was regarded as a consequence of adaptation probably involving long-term phenotypic or geonotypic change (Darchambeau and Thys 2005).

Significant ontogenic differences in P AE were found in our study, consistent with the observation by Hessen and Anderson (1990) that the juveniles of *Daphnia* had a higher biomass-specific uptake of P than had adults from bacteria. The juveniles have higher P requirements than the adults because they maintain high growth rates by synthesizing protein rapidly, during which a large amount of RNA is involved, consistent with the growth rate hypothesis (Sterner and Elser 2002). On the contrary, the modeling results by Wen et al. (1994) suggested that there was no correlation between body size and the P AE. Since six species at different life stages were included in their modeling study, it is difficult to conclude the interspecific and intraspecific differences in P requirements.

The P excretion rates of juveniles and adults were 12–63 ng P mg DW⁻¹ h⁻¹ and 8–33 ng P mg DW⁻¹ h⁻¹, respectively, at different food concentrations. When algae of different C:P ratios were fed to animals, the excretion rates were 3–57 ng P mg⁻¹ h⁻¹ and 1–18 ng P mg⁻¹ h⁻¹ in juveniles and adults, respectively. These rates were close to the low ends of the broad range of P release rates reported in the literature (20–1,100 ng P mg⁻¹ h⁻¹, Rigler 1961; Peters 1975; Wen et al. 1994). Generally, the excretion rate

was higher in juveniles than in adults, consistent with the prediction of the allometric relationship of the P release rate (Wen et al. 1994). Based on the equation constructed by Wen et al. (1994) (excretion rate = 0.021 W^{-0.30}), the juveniles and adults had P release rates of 32–52 ng P mg h⁻¹ and 27–36 ng P mg h⁻¹ under P sufficient conditions, respectively. These values were generally very close to our direct measurements.

The P excretion decreased dramatically with increasing dietary C:P ratio, comparable to the results found by Demott et al. (1998) and those predicted by stoichiometric models (Frost et al. 2004). However, the ratio of DP to ingestion was rather constant at different dietary C:P ratios, but Demott et al. (1998) found that the excreted fraction of ingested P decreased with increasing C:P ratios. In our study, the released DP was directly measured, but the P excretion in their study was obtained by calculation. Although *Daphnia* released similar fractions of ingested P under different dietary nutrient conditions, the absorption of P increased with an increase in dietary C:P ratio, and the DP/absorption thus decreased, suggesting that both absorption and excretion under P-deficient conditions are important for stoichiometric regulation. In long-term efflux experiments, the DP release was also considered as a compartment. It is notable that DP release during the long-term depuration period included both the excretion and molting fluids (Peters and Rigler 1973). However, the DP release by the neonates cannot be avoided, which may partially explain the higher DP release rates of adults in the long-term experiment than in the short-term experiments.

P turnover in Daphnia and loss budget—In this study, the turnover rate for body P in *Daphnia magna* was 10–30% of body P daily. Very little information on the P turnover in zooplankton can be directly extracted from the literature for comparisons with our measurements. Lehman and Naumoski (1985) reported a much higher turnover of the tissue P in *Daphnia pulex*. In their study, the daily turnover rates of *D. pulex* fed low-P algae were 50–67% and in those fed high-P algae, the rate increased to 360–500%. It may be possible to estimate roughly the turnover rate based on the measurements of DP excretion in *Daphnia*. For example, the excretion rate of P in *D. magna* was 32 ng P mg⁻¹ h⁻¹ (Rigler 1961), and the turnover rate was calculated to be 0.026 d⁻¹ with a mean P content of 18 µg P mg⁻¹ (Peters and Rigler 1973). However, this calculation only considered the P excretion into the dissolved phase, whereas other compartments of loss (such as molts and reproduction) also need to be considered. Similarly, with the wide range of release rates of 0.04–1.5 µg P mg DW⁻¹ h⁻¹ in other *Daphnia* species (summarized in Wen et al. 1994), it can be estimated that the P turnover rates in these species were 0.05–1.44 d⁻¹, assuming that the same specific P content is for all *Daphnia* and that the release is only by excretion.

In this present study, the turnover rate was independent of food quantity, but was significantly influenced by the food C:P ratios. Consistently, Lehman and Naumoski (1985) argued that the animals were able to retain body P to maintain a homeostatic stoichiometric state. However, when the changes in ingestion and absorption of animals

facing the dietary shift from the P-sufficiency to P-deficiency were combined, the increase in AE and the slow-down in turnover may not eventually avoid the decrease in P content in the animals because of the major decrease of P ingestion with increasing C:P ratio. Juveniles had significantly lower turnover rates of phosphorus compared with adults under different dietary C:P ratios. This may indicate the high demand of P and high retention of body P in juveniles, since they commonly have higher growth rates than adults.

To our knowledge, this study is the first to simultaneously quantify the P investments in molts, neonate production, and excretion in *Daphnia*. In our experiments, P loss by molting was more important than P loss by excretion and neonate reproduction. Shed molts respectively accounted for 27–39% and 26–33% of body P in juveniles and adults during the 3 days of depuration or, on average, 9–13% of the body P was lost from the animals per day. Considering that the normal interval between two molt sheddings is 2–3 days, our results were close to the findings by Vrede et al. (1999), who estimated that the carapace contained at least 14% of body P. In our study, the absolute loss rate by molting was 46–54 ng mg⁻¹ h⁻¹ and 88–98 ng mg⁻¹ h⁻¹ in juveniles and adults, respectively, under P-sufficient conditions. Based on the assumptions that (1) the maximum growth rate for juveniles was 0.5 day⁻¹; (2) the body C:P ratio was 30; and (3) the C content of *Daphnia* was 45% of dry weight (Hessen, 1992), the P loss by molting in juveniles with an average dry weight (DW) of 120 µg was 0.83 µg P under higher food levels. Therefore, the specific absolute P loss rate was 43.2 ng mg DW⁻¹ h⁻¹, which was also very close to our actual experimental results. Under the P-deficient condition, the P release by moulting decreased. Sterner et al. (1993) observed that the attachment of a molt to the posterior margin of new carapace caused difficulty in moulting in *Daphnia* fed P-deficient algae. It has also been suggested that some carapace-bound P may be lost during or after the molting process, which is accompanied by an increase of excretion (Scavia and McFarland 1982) and lower specific calcium in cast-off exuviae (Vrede et al. 1999). Thus, the P loss through molting may even be underestimated in our study.

P loss by neonate production played a less important role compared with molting. Vrede et al. (1999) found that 14% and 20% of body P was allocated to the molts and eggs, respectively, but there was a large variation in that study. As much as 35% of body P was reported to be lost in *Daphnia pulicaria* through reproduction by Ventura and Catalan (2005). In our study, assuming that 1.5% of dry weight (DW) was P, the averaged P loss through neonates release was 12% body P day⁻¹ under P-sufficient conditions, which then decreased to 3.2% body P day⁻¹ under P limitation, suggesting that reproduction was a significant drain of body P. The contribution of reproduction decreased with increasing dietary C:P ratio. As the numbers of neonates released were comparable among different food qualities (He and Wang unpubl. data), the neonates contained a higher P content in high-quality than in low-quality treatments, consistent with the finding by Boersma and Kreutzer (2002). Hessen and Rukke (2000)

estimated that the C:P ratio of an intact carapace was 65 by atoms, which was much lower than that of eggs (120, Færøvig and Hessen 2003), suggesting that the molts may be more vulnerable to P limitation.

To conclude, the dietary C:P ratio affected the AE, turnover rate, excretion rate, and molt loss in *D. magna*, and the food concentration affected the AE and excretion rate. As the most important compartment, molts may be a large drain on the carapace-bound P since *Daphnia* shed some 10–18 molts across a lifespan. Moulting may also contribute to the P sedimentation from the water column when the molts eventually settle. When phosphorus is limited, the animals decrease the P loss in the molts. However, the C:P ratio of molts under different food qualities needs to be further quantified. It is not clear how the animals recruit the element during the process of molting. P loss (investment) in reproduction decreased in the adults under P-limitation. Reproduction represented a considerable drain of P from the mother body and caused a variance in body stoichiometry. Further efforts are needed to clarify stoichiometric regulation of P in freshwater crustaceans.

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Received: 3 January 2006

Accepted: 28 August 2006

Amended: 20 September 2006